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
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
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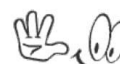
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# Effectiveness of Chlorogenic Acid Supplementation on VEGF Serum and Placental MAP Kinase Expression in Carbon Black-Exposed Pregnant *Rattus norvegicus*

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## ABSTRACT:

The aim of this study was to determine the effect of chlorogenic acid on the decrease of placental apoptosis in pregnant *Rattus norvegicus* exposed to carbon black by observing the expression of Mitogen Activating Protein Kinase (MAP Kinase) and Vascular Endothelial Growth Factor (VEGF) as indicators of apoptosis. This study was a laboratory experimental study, with five treatment groups and seven replications using female rats (*Rattus norvegicus*). The study was started by mating the female rats and exposing them to carbon black of 1064 mg/m<sup>3</sup> for 8 hours. Then, for MAP Kinase expression examination, immunohistochemical staining was performed and VEGF measurements were done using ELISA. Administration of chlorogenic acid at a dose of 13.4 mg/kg body weight in pregnant rats exposed to carbon black resulted in a decrease of MAP Kinase expression in placenta compared with a positive control group not exposed to carbon black. Carbon black-exposed pregnant rats treated with chlorogenic acid at a dose of 13.4 mg/kg BW showed increased VEGF levels compared with a positive control group not exposed to carbon black. In conclusion, the administration of chlorogenic acid may decrease MAP Kinase and increase VEGF levels.

**KEYWORDS:** Carbon black; MAP Kinase; VEGF; *Rattus norvegicus*.

## INTRODUCTION:

Carbon black is a cytotoxic, genotoxic and most stable particulate matter (PM) in the air in the form of carbon monoxide (CO) compounds. The amount of CO in the air is close to 164.383,56 mg/m<sup>3</sup> every day. This carbon source comes from factory exhaust gas, motor vehicle emissions, and domestic waste incineration. Some individuals may be exposed to the presence of carbon black in the working environment. Communities most frequently exposed to carbon black are factory workers, motor vehicle workshops, fuel industry, oil and coal industries.

The association of carboxyhemoglobin causes tissue and placenta hypoxia by expressing Hypoxia-Induced Factor-1 Alpha (HIF-1alpha) in order to decrease angiogenesis processes that may lead to impaired growth and development in the fetus, preterm birth and low birth weight (Rifatul, 2011). According to Gratton (2001), VEGF is one of the angiogenic cytokines secreted by various cells and serves as a survival factor for endothelial cells in vivo and in vitro. VEGF may also act as a mitogen and chemoattractant. In the process of angiogenesis, VEGF plays a role to repair damaged blood vessels, as well as for the process of feeding the fetus that passes through the blood vessels. Increased MAP Kinase p53 will cause disruption of the placenta, whose implementation will lead to premature birth. Increased cytokines may interfere with the differentiation of ectoderm, mesoderm and endoderm cells during the course of the pro-inflammatory organogenesis process. Disturbance of mesoderm cell differentiation may cause disturbance to bone growth (Gilbert, 1988). Antioxidant provision is one way to suppress free radicals. Coffee contains one substance that acts as an antioxidant. Green coffee beans (Coffee robusta Lindau.) are rich in polyphenols. Tannin is the main polyphenol content of coffee beans, while chlorogenic acid (CGA) is the main polyphenol in coffee beans. CGA content in arabica coffee is 4-8.4% and in robusta coffee is 7-14.4% (Thom, et al., 2007). According to EFSA (2011), the dose of CGA to prevent the occurrence of DNA damage, prevent apoptosis, and stabilize blood glucose levels of 9 mg/kg body weight. This study aims to determine the effective dose and antioxidant role contained in Chlorogenic Acid (CGA) in Green coffee bean (Coffea robusta Lindau) in improving body weight and fetal length, blood VEGF levels, and MAP Kinase expression.

## MATERIALS AND METHODS:

### Research procedure:

#### In vivo pregnancy induction:

The rats were injected with Pregnant Mare Serum Gonadotropin (PMSG) of 10 IU, then after 48 hours, injected with 10 IU of Human Chorionic Gonadotropin (HCG). Mating with male rats was performed with monomating method between one male rat and one female rat in a cage. Seventeen hours after being mated, the vaginal plug was examined. If the presence of vaginal plug was positive, the rat was considered as being pregnant on day zero and collected as a population of pregnant rats. If no vaginal plug was found, it was considered negative and not used as a sample.

#### Selection of carbon black:

Carbon black is carbon in powder form which has been widely used as a model for particles diesel emission without chemistry and metals. Some its chemical and physical features are similar to nano-based carbon engineering particles. Carbon black comprises carbon with impurities less than 1% organic and inorganic (Brown et al., 2000).

#### Population and sample:

This study was a true experimental laboratory study with Randomized Complete Randomized Design model which used rats consisting of 5 groups, with the number of replications per group of 6 rats each, with the following calculation:  $t(n-1)^2$  15; with  $t$ =number of treatments,  $n$ =number of replications (Kusniningrum, 2000). The negative control group comprised 6 normal rats without treatment (KN); positive control group: pregnant rats+receiving carbon black treatment and dissected on day 18 (KP); treatment group 1: 6 pregnant rats+carbon black exposure at 1064 mg/m<sup>3</sup> for 8 hours per day, at 6-17 days gestation and sacrifice on day 18+ receiving chlorogenic acid supplementation at 6-17 day gestation 4.5 mg/kg body weight (P1); and treatment group 2: 6 pregnant rats+carbon black exposure at 1064 mg/m<sup>3</sup> dose for 8 hours per day at 6-17 days gestation, and sacrificed on day 18+ receiving chlorogenic acid supplementation at 6-17 days gestation of 9 mg/kg body weight (P2); treatment group 3: 6 pregnant rats + carbon black exposure at 1064 mg/m<sup>3</sup> dose for 8 hours per day at 6-17 days gestation and sacrificed on day 18+, receiving chlorogenic acid supplementation at 6-17 days gestation by 13.5 mg/kg body weight (P3).

#### Measurements of serum VEGF levels with ELISA:

All reagents were left at room temperature (18-25°C) before use. Pipetted as much as 50  $\mu$ L standard, the samples were inserted into the microplate. A 100  $\mu$ L VEGF Enzym Conjugate was added for each microplate, then shaken for 30 seconds and incubated at 37°C for 1 hour. After incubation, the solution on the microplate was removed and then washed with a Washing Solution 250-300  $\mu$ L and shaking were performed for 3 minutes. Washing was repeated for 5 times. Then the solution was reversed, pressed firmly with absorbent paper and dried with a tissue. A 100  $\mu$ L substrate of TBM solution was added to each microplate according to the sequence. The tube was incubated for 10 minutes at room temperature and covered with film glass and then wrapped with aluminum foil. To stop the reaction, 50  $\mu$ L Stop Solution was added to each microplate gently. The mix was ?? shaken for 5 seconds. Then the microplate was fed into the ELISA Spectrophotometer, read and observed (Farah et al., 2006).

#### Examination of MAP kinase expression in placenta with immunohistochemistry:

The identification procedure of MAP kinase expression was performed by the immunohistochemical method in which the placenta preparations prepared on the glass object were dyed in xylol twice, stratified alcohols (100%, 90%, 80%, 70% and 30%) respectively, then washed in PBS with a pH of 7.4 3 times each for 5 min, immersed in 3% hydrogen peroxidase (H<sub>2</sub>O<sub>2</sub>) for 5-10 min, and immersed in 1% BSA in PBS for 10-30 min at room temperature. The primary antibody of MAP Kinase was added for 1 hour at room temperature, washed in PBS pH 7.4 for 3 times 5 minutes. SA-HRP (Strep Avidin-Horse Radish Peroxidase) was added for 30-60 minutes at room temperature, and washed in PBS pH 7.4 for 3x5 minutes. Chromogen DAB (3,3'-diaminobenzidine tetrahydrochloride) was added for 10-20 min and washed in PBS for 3 times 5 min at room temperature, then counterstain was added using methyl green for 3 min. Mounting was done with entellan. Observations were made using a microscope at 1000 magnification times. Determination of the amount of MAP Kinase expression was determined from the amount of brownish color change in placenta cells compared with the controls (Robyt and White, 1987; Aulanni'am 2004).

### RESULTS AND DISCUSSION:

#### MAP kinase expression on the placenta:

MAP Kinase expression in each sample was assessed semiquantitatively according to modified Remmele method (Novak et al., 2007). Remmele scale index (IRS) is the multiplication of immunoreactive cells percentage score that expresses MAP kinase with color intensity score generated on cells. The effect of carbon black exposure on placental MAP Kinase expression through immunohistochemical staining based on the dose of chlorogenic acid therapy was calculated semi quantitatively, and the analysis of MAP Kinase expression was done using Kruskal Wallis method, which can be seen in Table 1.

**Table 1. Expression of MAP kinase in exposed pregnant rat placenta**

Treatment	Positive percentage score (A)	Color intensity score (B)	Irs Index (AxB)	Mean Rank $\pm$ Standard Deviation

<b>KN</b> Pregnant rats without carbon black exposure	KN-1 KN-2 KN-3 KN-4 KN-5 KN-6 KN-7	2 1 0 0 1 1 1	1 1 0 0 0 1 1	2 1 0 0 0 1 1	7.36 ± 0.65 <sup>a</sup>
<b>KP</b> carbon black exposure 1064 mg/m <sup>3</sup> to pregnant rats aged 6-17 days for 8 hours/day	KP-1 KP-2 KP-3 KP-4 KP-5 KP-6 KP-7	3 3 2 2 1 1 2	3 3 3 3 3 3 1	9 9 6 6 3 3 2	28.50 ± 1.17 <sup>b</sup>
<b>P1</b> carbon black exposure 1064 mg/m <sup>3</sup> to pregnant rats aged 6-17 days for 8 hours/day + CGA dose 4.5 mg/kg BW	P1-1 P1-2 P1-3 P1-4 P1-5 P1-6 P1-7	2 3 3 3 1 3 2	3 3 2 2 3 1 1	6 9 6 6 3 3 2	27.93 ± 1.35 <sup>bc</sup>
<b>P2</b> carbon black exposure 1064 mg/m <sup>3</sup> to pregnant rats aged 6-17 days for 8 hours/day + CGA dose 9 mg/kg BW	P2-1 P2-2 P2-3 P2-4 P2-5 P2-6 P2-7	1 0 0 1 2 1 1	3 0 0 2 1 2 1	3 0 0 2 2 2 1	17.50 ± 0.68 <sup>c</sup>
<b>P3</b> carbon black exposure 1064 mg/m <sup>3</sup> to pregnant rats aged 6-17 days for 8 hours/day + CGA dose 13.5 mg/kg BW	P3-1 P3-2 P3-3 P3-4 P3-5 P3-6 P3-7	1 0 1 1 0 1 0	2 0 1 1 0 3 0	2 0 1 1 0 3 0	8.71 ± 0.71 <sup>a</sup>

**Notes:**

KN: Negative control, pregnant rats without exposure to carbon black and without Chlorogenic Acid therapy

KP: Positive control, pregnant rats exposed to carbon black without Chlorogenic Acid therapy

P1: Treatment group 1, pregnant rats exposed to carbon black with Chlorogenic Acid therapy at 4.5 mg/kg BW

P2: Treatment group 2, pregnant rats exposed to carbon black with Chlorogenic Acid therapy at 9 mg/kg BW

P3: Treatment group 3, pregnant rats exposed to carbon black with Chlorogenic Acid therapy at 13.5 mg/kg BW

**Notation:**a, b, c and d: difference of notation showed a significant difference between treatment groups ( $p < 0.05$ )

Table 1 shows that MAP Kinase expression in the negative control group (a group of pregnant rats without treatment of carbon black and without Chlorogenic Acid therapy) did not show significant results compared with P3 group (a group of pregnant rats exposed to carbon black, with Chlorogenic Acid therapy of 13.5 mg/kg BW) as indicated by a low percentage of MAP Kinase immunoreactive cell. Positive control group (a group of pregnant rats exposed to carbon black without Chlorogenic Acid therapy) and P1 group (pregnant rats, exposed to carbon black, and Chlorogenic Acid therapy of 4.5 mg/kg BW) showed higher immunoreactive cell percentage scores than negative controls, P3 and P2. P2 group (pregnant rats, with carbon black exposure, and Chlorogenic Acid therapy of 9 mg/kg BW) showed MAP Kinase immunoreactive cell percentage score lower than positive control group and P1, but MAP Kinase immunoreactive cell percentage score in P2 was higher than those in P3 and negative control.

Low MAP Kinase immunoreactive score in placenta shows the intensity of light brown color in immunoreactive placenta cells. High immunoreactive MAP Kinase score in placenta shows dark brown. The expression placenta MAP Kinase in various treatment groups can be seen in Figure 1.

**Figure 1. Differences in MAP Kinase expression on trophoblast cells (arrows) of placenta between treatments.**

The above slide shows that MAP Kinase expression in P3 group is weaker than those in other treatment groups (KP, P1, and P2). The above slide also shows that the P3 immunoreactive cell expression was almost the same as that in the negative control group (immunohistochemical staining, 1000x magnification, Nikon H600L microscope, DS Fi2 300 megapixel camera).

Carbon black is the most widely used nanomaterial and an exhaust gas of motor vehicles, which is cytotoxic and genotoxic. Carbon black is known to affect the fetus through several mechanisms, including directly entering the placenta, causing a change in placental function, and indirectly affecting the cytokine changes present in the placenta through the respiratory process

(Hougraad et al., 2011).

Exposure to carbon black in pregnant rats may lead to the intrauterine fetus to produce carbon monoxide in the body with higher levels. The high exposure to carbon monoxide produced by carbon black during the burning process may increase the bond between hemoglobin and carbon monoxide that produces carboxyhaemoglobin. The binding of carboxyhemoglobin may cause tissue and placental hypoxia by expressing Hypoxia Induced Factor-1 Alpha (HIF-1alpha). Exposure to hydrocarbons affects the morphological and physiological responses of the rats placenta, especially in placental labyrinthine, the site of encounter of parental and fetal blood circulation. The presence of hydrocarbon exposure causes high ROS in placental cells, including endothelial blood vessels. High ROS causes both necrosis and apoptosis in endothelial cells. At placental level, oxidative stress occurring during pregnancy will induce macrophages to produce pro-inflammatory cytokines by modulating the transcription of pro-inflammatory genes through a phosphorylation cascade using MAP Kinase.

Chlorogenic acid is an antioxidant that inhibits increased ROS in tissues and prevents tissue hypoxia, thereby directly decreasing MAP Kinase expression in placenta. Administration of chlorogenic acid therapy at 13.5 mg/kg BW may decrease MAP Kinase expression in order to prevent excessive placental apoptosis, to prevent premature abortion and to improve the performance of pregnant rats.

#### VEGF levels:

Cytotoxic and genotoxic carbon black exposure may affect the angiogenesis process in the placenta of pregnant rats during pregnancy of 6-17 days and will eventually lead to increased apoptosis. The analysis used on the VEGF level was carried out in a depressive manner. Table 2 shows the mean and standard deviations in various treatment groups (KN, KP, P1, P2, and P3).

**Table 2. VEGF levels in pregnant rats exposed to carbon black without and with chlorogenic acid therapy**

Groups	Mean $\pm$ Standard Deviation
Control Negative	192.33 $\pm$ 14.84
Control Positive	162 $\pm$ 36.98
Treatment 1	231 $\pm$ 36.94
Treatment 2	249 $\pm$ 36.4
Treatment 3	263.16 $\pm$ 21.65

Table 2 shows the mean difference between treatments. The lowest mean VEGF level was shown in the positive control group (KP), while the highest mean in treatment group 3 (P3) that received carbon black exposure and CGA supplementation in a dose of 13.5 mg / Kg BW. This can also be seen in Figure 2.

**Figure 2. VEGF in pregnant rats exposed to carbon black without and with chlorogenic acid therapy.**

Figure 2 shows high VEGF levels in P3, P2, P1, and KN compared to KP. This suggests that chlorogenic acid has an effect on the increase of VEGF levels, which indicates that high levels of VEGF are able to regenerate in the blood vessel system to prevent apoptosis. Carbon black exposure given to pregnant rats causes cytokine changes in the placenta that will affect cellular response. This condition will contribute to inflammation and disruption of homeostasis tissue, which will continuously affect the vascular system in the placenta. Endothelial blood vessel cells, located along the vascular tract in the placenta, are impaired. As a result of inflammation, existing cytokines will contribute to pathological processes, such as angiogenesis, vascular homeostasis, and ischemia (Monique et al., 2001).

VEGF acts as a pro-inflammatory cytokine by increasing endothelial cell permeability, stimulating endothelial expression, molecular adhesion, and having the ability to act as a monocyte chemoattractant. VEGF is widely expressed by epidermal keratinocytes cells for wound healing and psoriasis, characterized by increased microvascular permeability and angiogenesis. One of the most influential cytokines in the formation of new blood vessels is VEGF. There are many factors that affect VEGF expression, one of which is the hypoxic stimulus (Levy et al., 1995). Other factors, such as cytokines, oncogenes, NO, and protein modulators kinase c, were reportedly capable of stimulating VEGF production (Brown et al., 1997). It was also reported that MAP Kinase and phosphatidylinositol-3-kinase (PI3-kinase) are involved in VEGF induction by growth factors, cytokines, and hypoxia (Clarke et al., 2001; Jiang et al., 2000).

In pregnant rats exposed to carbon black, the vascularization regeneration system of the rat placenta is quite high and is functioned to withstand the inflammatory invasion, since rats' placenta type has highly complex branching in the placental labyrinth area with the intention that the exchange of nutrition between the mother and the fetus is efficient (Watson and Cross, 2005). Pregnant rats exposed to carbon black and supplemented with Chlorogenic Acid in a dose of 13.5 mg/kg showed a good regeneration of



vascularization with an indication of high levels of VEGF that resist inflammatory invasion, while maintaining nutrient exchange, and preventing excessive apoptosis in the placenta at the early age of pregnancy so that it can prevent the occurrence of early abortion.

### CONCLUSION:

The administration of Chlorogenic Acid at a dose of 13.4 mg/kg body weight in pregnant rats exposed to carbon black may decrease MAP Kinase expression and increase VEGF levels in the placenta compared with positive control group not receiving Chlorogenic Acid.

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